Progressive pulmonary fibrosis is mediated by TGF-β isoform 1 but not TGF-β3

Kjetil Ask [a], Philippe Bonniaud [a,b], Katja Maass [c], Oliver Eickelberg [c],
Peter J. Margetts [a], David Warburton [d], John Groffen [d],
Jack Gauldie [a], Martin Kolb [a,e,*]

[a] *Department of Pathology and Molecular Medicine, Center for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada*
[b] *Service de Pneumologie et Réanimation Respiratoire, CHU du Bocage et Université de Bourgogne, Dijon, France*
[c] *University of Giessen Lung Center, Department of Medicine II, Giessen, Germany*
[d] *Developmental Biology Program, Saban Research Institute, Children's Hospital Los Angeles,
University of Southern California, United states*
[e] *Department of Medicine, Firestone Institute for Respiratory Health, McMaster University,
50 Charlton Ave East, Room T2121, Hamilton, Ontario, Canada*

Abstract

Tissue repair is a well-orchestrated biological process involving numerous soluble mediators, and an imbalance between these factors may result in impaired repair and fibrosis. Transforming growth factor (TGF)-β is a key profibrotic element in this process and it is thought that its three isoforms act in a similar way. Here, we report that TGF-β3 administered to rat lungs using transient overexpression initiates profibrotic effects similar to those elicited by TGF-β1, but causes less severe and progressive changes. The data suggest that TGF-β3 does not lead to inhibition of matrix degradation in the same way as TGF-β1, resulting in non-fibrotic tissue repair. Further, TGF-β3 is able to downregulate TGF-β1-induced gene expression, suggesting a regulatory role of TGF-β3. TGF-β3 overexpression results in an upregulation of Smad proteins similar to TGF-β1, but is less efficient in inducing the ALK 5 and TGF-β type II receptor (TβRII). We provide evidence that this difference may contribute to the progressive nature of TGF-β1-induced fibrotic response, in contrast to the limited fibrosis observed following TGF-β3 overexpression. TGF-β3 is important in "normal wound healing", but is outbalanced by TGF-β1 in "fibrotic wound healing" in the lung.
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1. Introduction

After lung injury, acute inflammation and tissue repair represent key mechanisms engaged to halt the injurious stimulus, remove infectious organisms, and initiate immediate repair to crucial membranes that function to provide gas exchange for survival. In most cases, this results in reconstitution of normal lung function. In

chronic tissue injury with repeated episodes of inflammation, however, many of the control mechanisms involved in this otherwise well-orchestrated process are bypassed and injurious stimuli persevere. This leads to remodeling of lung tissue characterized by distorted matrix deposition, mesenchymal cell proliferation, and alteration of normal lung structure with compromised gas exchange, a process known as pulmonary fibrosis (PF) (Noble & Homer, 2004; Selman, King, & Pardo, 2001; Ward & Hunninghake, 1998).

Several growth factors are instrumental to the initiation as well as the termination of such inflammatory and remodeling processes, which have been studied extensively in the context of PF (Kelly, Kolb, Bonniaud, & Gauldie, 2003). It is likely that dysregulations in the balance of these growth factors play a major role in determining the differences between normal and pathologic tissue repair. Among these, transforming growth factor (TGF)-β is one of the key cytokines involved in the pathogenesis of PF (Leask & Abraham, 2004). TGF-β is the prototype of a group of polypeptide growth factors, which exert multiple effects in all cell types studied thus far (Letterio & Roberts, 1998). In mammals, three closely related isoforms with 64–85% amino acid sequence homology are described, which exhibit a substantial overlap in biological functions. Most publications investigating tissue fibrosis have focused on the most prominent isoform, TGF-β1, demonstrating an array of profibrotic functions. It is well established that TGF-β1 promotes differentiation of fibroblasts into activated myofibroblasts, enhances collagen synthesis, and reduces collagen degradation by downregulation of proteases and upregulation of protease inhibitors (Kelly et al., 2003). Only few studies have specifically addressed the role of TGF-β3 in the pathogenesis of pulmonary fibrosis. Moreover, the balance amongst the TGF-β1 and -β3 isoforms is neglected, but important component in the process of tissue repair. Some of these studies supported no major individual role, and claimed that TGF-β3 acts in concert with TGF-β1 (Eickelberg et al., 1999; Khalil, Shing, & Whitman, 1993; Santana, Saxena, Noble, Gold, & Marshall, 1995). In contrast, data from targeted gene knockouts and experimental models of cutaneous wound healing and chronic inflammatory bowel disease suggested distinct features of TGF-β3, when compared with TGF-β1 (Ingman & Robertson, 2002; McKaig et al., 2002; McKaig, Hughes, Tighe, & Mahida, 2002; Shah, Foreman, & Ferguson, 1995; Van Themsche, Mathieu, Parent, & Asselin, 2007). Fetal wounds, which contain primarily TGF-β3, heal without scars, whereas adult wounds, which contain mainly TGF-β1 and -β2, always exhibit some degree of scarring

(Nath, LaRegina, Markham, Ksander, & Weeks, 1994). These observations have raised considerable research efforts in the field of cutaneous wound healing, as well as debates whether TGF-β3 might even carry anti-scarring properties (Shah et al., 1995).

Here, we report that TGF-β3 administered to rat lungs using transient overexpression initiates initial profibrotic effects similar to those elicited by TGF-β1, such as myofibroblast differentiation and increased ECM synthesis. However, TGF-β3 causes a lower intensity and duration of these effects and appears not to inhibit matrix degradation in the same way as TGF-β1. We also show that TGF-β3 overexpression results in an upregulation of Smad proteins similar to TGF-β1, but is less efficient in inducing expression of the ALK 5 and TGF-β type II receptor (TβRII). Examination of Smad-independent pathways shows that TGF-β1-treated tissues have lower levels of phosphorylated AKT compared to TGF-β3 treated animals, indicating a possible protective mechanism. We provide evidence that this difference may contribute to the progressive nature of TGF-β1-induced fibrotic response, which contrasts the limited fibrosis observed following TGF-β3 overexpression. We thus hypothesize that TGF-β3 is important in "normal wound healing", but is outbalanced by TGF-β1 in "fibrotic wound healing".

2. Methods

2.1. Recombinant adenovirus

For construction of AdTGFβ3 a mutated mouse TGF-β3 cDNA was used. Cys223, 225Ser mutations were generated using an overlap extension method. Mutated and wild-type cDNAs were subcloned into a pSG5 vector and subsequently transfected into COS-1 cells. Supernatants were used for a PAI-1/luciferase assay: without heat activation, the mutated TGF-β3 cDNA produced biologically active growth factor several times more active than a wild-type control. With heat activation, no significant differences were found between mutated and wild-type samples (data not shown). The plasmid with the mutation was cloned into a p73 shuttle vector with a human cytomegalovirus (CMV) promoter and co-transfected into COS-1 cells. Similarly, a replication-deficient adenovirus carrying a mutated TGF-β1 gene was constructed as previously described (Sime, Xing, Graham, Csaky, & Gauldie, 1997). The resulting replication-deficient virus was amplified and purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography, and plaque titred on 293 cells. The control vectors, AdDL, with no

insert in the deleted E1 region were produced in the same way.

2.2. Cell culture and bioassay for TGF-β

A549 cells were infected with AdTGF-β1$^{223/225}$, AdTGF-β3$^{223/225}$ or AdDL70 at a multiplicity of infection (MOI) of 1, 10 and 100 PFU/cell similar to earlier studies. Bioactive TGF-β was detected by an established bioassay using Mink lung epithelial cells (MLEC clone 32, kindly provided by D. Rifkin, New York, NY, USA), with a stable transfection of an 800-bp fragment of the 5′ end of the human plasminogen activator inhibitor 1 (PAI-I) gene fused to the firefly luciferase reporter gene. D-(−)-Luciferin and the firefly luciferase standard (Boehringer, Mannheim, Germany) were used and assayed by luminometer (Lumat LB 9501; Berthold Systems, Pittsburgh, PA, USA). Data are presented in relative light units (RLU).

2.3. Animal treatment

Female Sprague–Dawley rats (200–250 g bodyweight) received AdTGF-β1 (5×10^8 plaque forming units [pfu]), AdTGF-β3 or AdDL (both 1×10^9 pfu) in a volume of 50 µl phosphate-buffered saline (PBS) by intratracheal administration during a small surgical procedure. Rats were sacrificed at days 3, 7, 14, 28, and 60 after adenoviral administration by abdominal aorta bleeding. In another set of experiments we administered both AdTGF-β1 and -β3 concomitantly or each vector combined with AdDL to investigate for potential antifibrotic properties of TGF-β3 in this experimental setting. Bronchoalveolar lavage (BAL) was then performed as described previously. For histological examination, lungs were inflated and fixed by intratracheal instillation of 10% neutral buffered formalin at a constant pressure of 20 cm of water for 5 min. For RNA after washing with PBS, the lung was removed and frozen immediately in liquid nitrogen. Frozen tissue samples were ground and stored at −70 °C until further processing. Rodent laboratory food and water were provided ad libitum. The animals were treated in accordance with the guidelines of the Canadian Council of Animal Care. All animal proce-dures were performed under inhalation anaesthesia with isofluorane (MTC Pharma, Cambridge, Ont., Canada).

2.4. Determination of cytokine levels in BAL fluid

Bioactive TGF-β1 and -β3 were detected in BALF using the MLEC bioassay as described above. A human TGF-β1 ELISA (R&D Systems, Minneapolis, MN, USA) was used to determine TGF-β1, according to the manufacturer's protocol.

2.5. RNA isolation and RT–PCR

RNA (1 µg) was DNase treated and then reverse tran-scribed (Invitrogen). Real-time quantitative polymerase chain reaction (PCR) analysis (TaqMan, Applied Biosystems, Foster City, CA, USA) was carried out using an ABI Prism 7700 Sequence Detector. Negative control samples (no template or no reverse transcriptase) were run concurrently. Results were normalized to β-2 microglobulin. Data were normalized to control treatment (AdDL) or naive animals. The sequences of primers and probes are shown in Table 1.

2.6. Western blot analysis

Frozen lung tissues were homogenized in liquid nitrogen and proteins isolated in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate and 1 mM β-glycerophosphate). The protease inhibitor cocktail Complete™ at a dilution of 1:25 (Roche Molecular Bio-chemicals, Indianapolis, IN, USA) and the phosphatase inhibitor Na$_3$VO$_4$ dilution 1:100 were added to the lysis buffer immediately prior to isolation. Protein concentra-tions in tissue lysates were determined using the Quick Start Bradford Protein Assay (BIO-RAD) and FusionTM Spectrophotometer (Packard). Equal amounts of pro-tein (50 µg) from each sample were then separated on 10% polyacrylamide SDS-PAGE gels and transferred to 0.2 µm nitrocellulose membranes (BIO-RAD). Mem-branes were blocked with 5% nonfat dry milk in PBST (PBS + 0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with antibodies listed in Table 2, followed by incubation with HRP-conjugated

Table 1
Taqman primers and probes

	Forward primer	Reverse primer	Probe
TIMP-1	GAACCGCAGCGAGGAGTTT	GGCAGTGATGTGCAAATTTCC	TCATCGCGGGCCGTTTAAGGAAT
TGF-β1	AAACGGAAGCGCATCGAA	GGGACTGGCGAGCCTTAGTT	CCATCCGTGGCCAGATCCTGTCC
β2-Microglobulin	CCGATGTATATGCTTGCAGAGTTAA	CCAGATGATTCAGAGCTCCATAGA	CACGTCACTCTGAAGGA

Table 2
List of antibodies and dilutions

Antibody	Company	Dilution
CDK4—rabbit polyclonal	Santa Cruz, CA, USA	1:1000
TGF-βRI—rabbit polyclonal	Santa Cruz, CA, USA	1:500
TGF-βRII—rabbit polyclonal	Santa Cruz, CA, USA	1:500
P-SMAD2 rabbit monoclonal	Cell Signalling Technology, MA, USA	1:1000
P-SMAD3—rabbit polyclonal	Cell Signalling Technology, MA, USA	1:500
P-AKT—rabbit monoclonal	Cell Signalling Technology, MA, USA	1:1000
AKT—rabbit polyclonal	Cell Signalling Technology, MA, USA	1:1000
P-p44/42 MAPK—mouse polyclonal	Cell Signalling Technology, MA, USA	1:1000
p44/42 MAPK—rabbit polyclonal	Cell Signalling Technology, MA, USA	1:1000

secondary antibodies at room temperature for 1 h. Antibody binding was detected by SuperSignal WestPico chemiluminescent substrate (Pierce Biotechnology Inc., Rockford, IL, USA).

2.7. Histology

After fixation in 10% buffered formalin for 24 h, lungs were paraffin embedded, sectioned, and stained with either hematoxylin & eosin, Masson's trichrome or Picro Sirius Red. Four axial sections of the distal left lung were stained with Picro Sirius Red (PS Red) and 20 microscopically fields were examined for pulmonary fibrosis by three blinded investigators. Each field was individually assessed for the severity of interstitial fibrosis using Ashcroft et al.'s semi-quantitative grading previously described (Ashcroft, Simpson, & Timbrell, 1988). Immunohistochemistry was carried out using antibody to αSMA (M0851, Dako Canada Inc., Mississaugua, Ont., Canada), phospho-Smad2 Cat no. 3101 (Cell Signaling Technology, Danvers, MA, USA) or TGF beta Receptor I (V-22) Cat No sc-398 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

2.8. Hydroxyproline assay

Frozen lung samples were homogenized in 5 ml deionized water. The homogenate was diluted (10×) and 1 ml of this suspension was hydrolyzed in 2 ml 6N HCl for 16 h at 110 °C. Hydroxyproline content was determined by a colorimetric assay described previously (Woessner, 1961). The results were calculated as micrograms of hydroxyproline per milligram wet lung weight using hydroxyproline standards (Sigma Chemicals).

2.9. Statistical analysis

Data are shown as mean ± S.E.M. For evaluation of group differences, we used the two-way ANOVA with Bonferroni post-tests (GraphPad Prism® 4.0) and Student's t-test. p values less than 0.05 were considered significant.

3. Results

3.1. Bioactivity of TGF-$\beta1$ and -$\beta3$ following transient overexpression in cell culture and in rat lungs

The efficacy of the AdTGF-$\beta3$ construct to transfect cells in vitro and lungs was confirmed by Northern blot (Fig. 1A). In vitro, AdTGF-$\beta3$ needed a higher number of virus particles per cell as quantified by multiplicity of infection to generate the same level of bioactive TGF-β in the PAI-1-luciferase cell assay (Fig. 1B). This is probably related to vector-specific issues (such as efficacy of gene transfer into the cells, utilization of the CMV promoter, length of inserted cDNA, etc.), and was the rationale for using 5×10^8 pfu AdTGF-$\beta1$ and comparing it to 1×10^9 pfu AdTGF-$\beta3$ and 1×10^9 pfu AdDL. These plaque forming units of viral vector resulted in the same levels of bioactive TGF-β in the BALF of both AdTGF-$\beta1$ and -$\beta3$ treated rats (Fig. 1C).

The bioactive transgenic proteins were undetectable in BALF after day 14 post-transfection. There was no TGF-$\beta1$ detectable in AdTGF-$\beta3$ or AdDL-treated animals as measured by ELISA (data not shown).

3.2. Progressive lung fibrosis in response to TGF-$\beta1$ overexpression as opposed to partially reversible fibrosis following TGF-$\beta3$

Both AdTGF-$\beta1$ and -$\beta3$ resulted in a similar mild inflammatory response with mainly mononuclear cells by day 7 (not shown). Fibroblast proliferation and accumulation of αSMA-positive, spindle-shaped cells as indication for myofibroblast differentiation was seen as early as 7 days post injection of adenoviral vectors. There
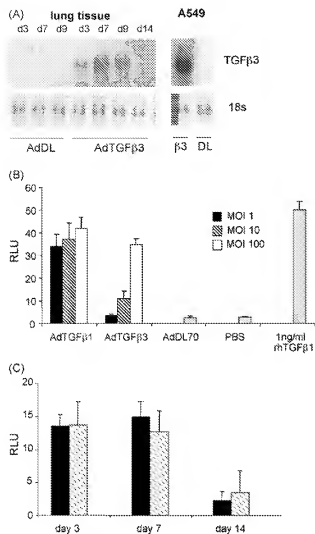
Fig. 1. (A) Transgenic TGF-β3 mRNA is detectable by Northern blot in cell culture (A549 cells) and rat lung tissue after exposure to AdTGF-β3 but not in control AdDL. (B) The bioactivity of transgenic proteins TGF-β1 and -β3 was demonstrated and compared to recombinant TGF-β by assaying supernatant of A459 cells (transfected with different concentration of adenovirus, MOI: multiplicity of infection) using mink lung epithelial cells stably transfected with a PAI-1-responsive luciferase gene (RLU = relative light units). (C) Bioactivity of TGF-β1 (solid) and TGF-β3 (shaded bars) in BAL fluid of rats exposed to AdTGF-β1 and -β3.

was no obvious difference between AdTGF-β1 and -β3. By day 14, collagen deposition was substantial and similar in lungs following overexpression of either TGF-β isoform (Fig. 2A–D). No fibroblast proliferation and collagen deposition was seen in control rat lungs (not shown). By day 28, histology showed severe fibrotic changes in the lungs of rats treated with AdTGF-β1 compared to AdTGF-β3 (Fig. 2E and F). Histological evidence of marked pulmonary fibrosis was still present in AdTGF-β1 exposed rats by day 60, but was almost absent in the AdTGF-β3 group (Fig. 2G and H).

Hydroxyproline analysis of lung homogenates as indication for collagen accumulation show an approximately twofold increase of hydroxyproline in AdTGF-β1 and -β3 treated rats compared to controls by days 14 and 28 with no difference between the isoforms ($p < 0.001$ versus AdDL, Fig. 3). By 60 days, the hydroxyproline concentration in AdTGF-β1 animals further increased to $5.2 \pm 0.3 \,\mu g/mg$ lung tissue, while it decreased to $3.0 \pm 0.3 \,\mu g/mg$ in AdTGF-β3 rats ($p > 0.001$).

3.3. Induction of TGF-β types I and II receptor and non-Smad proteins differ between AdTGF-β1 and -β3

Western blot was used to investigate Smad proteins, non-Smad-signalling pathways and TGF-β receptors in whole lung tissue by 7 days. There was no difference in the level of phosphorylation of Smad2 and 3 between the isoforms (Fig. 4A). Phospho-p44/42 and p44/42 was reduced in AdTGF-β1-treated and AdTGF-β3-treated animals compared to control ($p < 0.05$, Fig. 4B) with a trend to lower level in AdTGF-β1 versus AdTGF-β3. Similarly, phosphorylated AKT appeared to be reduced in animals receiving AdTGF-β1 compared to TGF-β3 and control (densitometry did not reach statistical significance, Fig. 4B). The TGF-β receptor I (ALK-5) and TGF-β receptor II were induced 7 days after adenoviral gene transfer, and most importantly, AdTGF-β1 caused a significantly stronger induction of ALK-5 and TGF-β receptor II compared to AdTGF-β3 (Fig. 4A). The activation of phospho-Smad 2 and expression of TGFβ RI in lung tissue was also assessed by immunohistochemistry. There was no difference in phospho-Smad 2 between AdTGF-β1 and -β3, positive cells were mainly bronchial and alveolar epithelium. TGFβ RI immunostaining was stronger in AdTGF-β1 exposed alveolar epithelial cells (Fig. 5).

3.4. AdTGF-β3 reduces AdTGF-β1-induced expression of TGF-β-responsive genes

Gene expression of TGF-β-responsive genes was examined in lungs following concomitant administration of AdTGF-β1 plus AdTGF-β3 and compared to AdTGF-β1 or AdTGF-β3 plus AdDL. The expression levels of TIMP-1 and TGF-β were elevated in lungs that had received AdTGF-β1 plus AdDL at both days 14 and 28 compared to naive lungs (Fig. 6A and B). The addition of AdTGF-β3 to AdTGF-β1 prevented the increase of TIMP-1, TGFβ1 (Fig. 6A and B) and other fibrogenic genes like Col1A2, Fibronectin-1 and
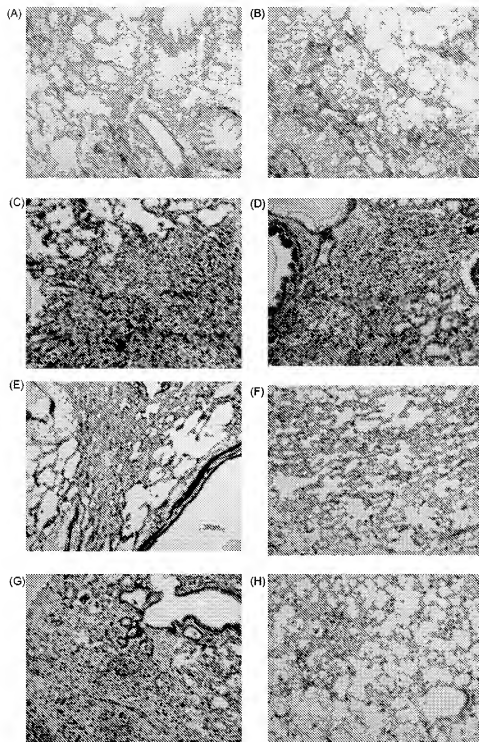
Fig. 2. Histology of lungs exposed to AdTGF-β1 (left panel) and AdTGF-β3 (right panel) at different time points. Accumulation of αSMA-positive, spindle-shaped cells as indication for myofibroblast differentiation (A and B, immunohistochemistry for αSMA) and substantial collagen deposition (C and D, Masson's trichrome) with no obvious difference between AdTGF-β1 and –β3 by day 14. No fibroblast proliferation and collagen deposition was seen in control rat lungs (not shown). (E and F, Masson's trichrome) By day 28, histology showed similar fibrotic changes in the lungs of rats treated with AdTGFβ1 compared to AdTGFβ3. (G and H, Masson's trichrome) Marked pulmonary fibrosis was still present in AdTGF-β1 exposed rats by day 60, but was almost absent following AdTGF-β3.

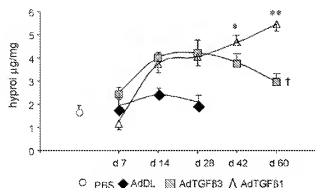Fig. 3. Lung collagen content as measured by hydroxyproline assay in rats exposed to AdTGF-β1, AdTGF-β3, empty vector AdDL and saline (PBS) over a period of 60 days ($p < 0.001$ for AdTGF-β1, AdTGF-β3, days 14, 28 and 42 vs. AdDL; *, $p < 0.01$ and **, $p < 0.001$ vs. AdTGFβ3; †, not significant vs. AdDL days 7–28).

CTGF by day 14 (not shown). By day 28, the AdTGF-β1-induced TIMP-1 and TGFβ1 gene expression was still suppressed when AdTGF-β3 was co-administered (Fig. 6A and B). Despite this clear negatively regulating effect of AdTGF-β3 on AdTGF-β1-induced gene expression, we were not able to prove a substantial antifibrotic effect of TGF-β3 in this model (Fig. 6C).

4. Discussion

TGF-β is a key cytokine in the pathogenesis of fibroproliferative disorders of the lung, kidney, liver, or skin (Wynn, 2004). In mammals, three closely related TGF-β isoforms have been cloned and characterized, exhibiting
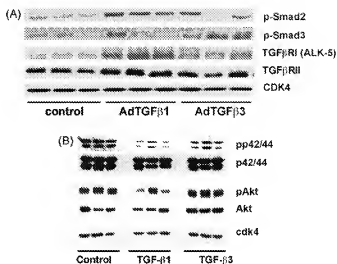

Fig. 4. (A) Western blot analysis confirmed this difference between AdTGF-β1 and -β3 7 days after administration of AdTGF-β1, -β3 and AdDL by detection of more TGF-βRI (ALK-5) and TGF-βRII protein in rat lung homogenate ($p < 0.01$ as quantified by band densitometry). (B) Smad-independent pathways were investigated by Western blots and showed a trend to lower activation levels in the p42/44 and the Akt pathways in AdTGF-β1-treated lungs compared to controls and AdTGF-β3 (bands represent different animals, $n = 3$).

64–85% amino acid sequence homology and a substantial overlap in biological functions (Leask & Abraham, 2004). Most studies thus far have focussed on the most prominent isoform, TGF-β1, and demonstrated an array of profibrotic functions. Only a limited number of studies have specifically addressed the role of TGF-β3 in the pathogenesis of wound healing and fibrosis (Khalil,

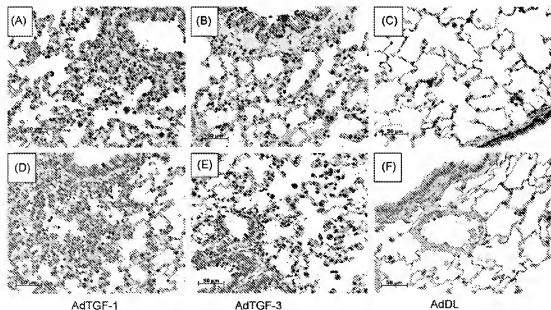Fig. 5. Immunohistochemistry showed no difference for phospho-Smad 2 between AdTGF-β1 and -β3 (A and B) but a stronger positivity for AdTGF-β1 exposed TGF-β RI in alveolar epithelial cells (D and E) at day 7 (C and F: control vector).

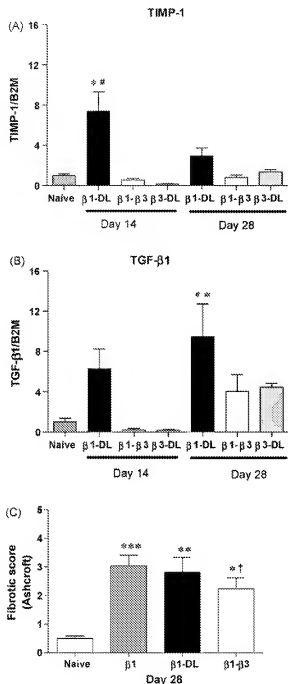Fig. 6. Gene expression of TGF-β1 and the protease inhibitor TIMP-1 in lungs of rats 14 and 28 days after exposure to AdTGF-β1 and -β3 in combination or combined with AdDL (measured by real-time RT-PCR). AdTGF-β1 induced a strong expression of both these genes when combined with AdDL. However, the gene induction following AdTGF-β1 when combined with AdTGF-β3 was significantly weaker at days 14 and 28 for TIMP-1 (A) and at day 14 for TGF-β1 (B) (*, different from naive $p < 0.05$ and #, different to AdTGF-β1 plus AdTGF-β3, day 14, ANOVA). (C) Fibrotic score obtained on naive animals on day 28, AdTGF-β1 (B1), AdTGF-β1 + AdDL (β1-DI) and AdTGF-β1 + AdTGF-β3 (β1–β3). *, $p < 0.05$; **, $p < 0.01$; ***, vs. naive †, not significant vs. AdTGF-β1 + AdDL, ANOVA).

O'Connor, Flanders, & Unruh, 1996; Khalil et al., 2001; Santana et al., 1995; Shah et al., 1995). While some of these studies assigned no major individual role to TGF-β3, there is also evidence for distinct and specific features of TGF-β3 that even suggest potential anti-scarring properties. One of the key reports supporting this idea is the observation that cutaneous wounds heal without or only limited scarring, when TGF-β3 is present in the wound bed, either constitutively as found in the embryonic/fetal state or delivered as a therapeutic agent (Nath et al., 1994; Shah et al., 1995). Similarly, cutaneous wounds heal without or only limited scarring when TGF-β1 or -β2 is antagonized by antibodies (Shah, Foreman, & Ferguson, 1992). In contrast, there is marked scarring in the absence of TGF-β3 and presence of high levels of TGF-β1 and -β2 (Hosokawa, Nonaka, Morifuji, Shum, & Ohishi, 2003; Kohama, Nonaka, Hosokawa, Shum, & Ohishi, 2002; Shah et al., 1995). We thus sought to define the role of the TGF-β3 in the development of PF in comparison with TGF-β1, and analyze potential antifibrotic properties of TGF-β3 in this devastating disease.

In this study, we have employed adenoviral gene transfer to induce transient overexpression of TGF-β3 in the lungs of rats after intratracheal injection and compared this to overexpression of TGF-β1. The transgenic protein were routinely detectable in BALF for at least 7 days, and the bioactivities of TGF-β1 and -β3 were not found to be different between the two isoforms (Fig. 1). TGF-β3 mRNA was not found in lung tissue beyond day 14 in AdTGF-β3-treated animals similar to studies previously with AdTGF-β1. Thus, it is unlikely that differences in the duration of exogenous gene expression are a major contributing factor to the observations reported here (Warshamana et al., 2002). AdTGF-β3 induced a rapid response including hypertrophy of alveolar epithelial cells and appearance of myofibroblasts, as early as 4 days after instillation of the adenovirus. Of note, this response was devoid of a major inflammatory component as assessed by histological examination. By day 14, we observed substantial fibrosis and collagen accumulation similar to animals exposed to AdTGF-β1. However, in contrast to AdTGF-β1 and despite the comparable bioactivity, the fibroproliferative response in AdTGF-β3-treated rats was transient in nature, and partially resolved after 60 days. We have recently published similar findings after overexpression of CTGF (connective tissue growth factor), which caused marked but transient fibrosis in the lung (Bonniaud et al., 2003). At the level of gene expression, we noticed that the duration of TIMP-1 upregulation following AdTGF-β3 was transient and returned to baseline by day 14, while this gene was persistently induced in AdTGF-β1-treated

rats. These findings strongly support previous reports showing that transient fibrosis in the lungs following CTGF overexpression or using bleomycin or AdTGF-β1 in fibrosis-resistant rats were accompanied by low levels of TIMP-1 gene induction (Bonniaud et al., 2004; Kolb et al., 2002).

Several studies have examined the ability of TGF-β isoforms to stimulate lung fibroblasts *in vitro*, but available data comparing the fibrogenic potential of the three TGF-β isoforms are inconclusive. In human fibroblast, both isoforms were reported to equally induce collagen, TIMP-1 and MMP-1 while inhibiting TIMP-2 (Eickelberg et al., 1999). Others have shown a differential expression pattern of protease and antiprotease levels. TGF-β3 alone was not sufficient to induce TIMP-1 expression in intestinal myofibroblasts (McKaig, McWilliams, Watson, & Mahida, 2003) or human endometrial carcinoma cells (Van Themsche et al., 2007). This is in line with a previous report indicating that TGF-β1 is a more potent inductor of TIMP-1 compared to TGF-β3 in human lung fibroblasts, which is consistent with the findings of our study (see Fig. 8 in Eickelberg et al., 1999). Further, exogenous TGF-β3, but not TGF-β1 increased MMP-9 expression and activity in cancer cells and a lip wound healing model (Hosokawa et al., 2003; Van Themsche et al., 2007), indicating a role in balancing protease/antiprotease levels. However, there is also a study suggesting that TGF-β3 may be an even more potent inducer of procollagen and inhibitor of matrix degrading activity than TGF-β1 and -β2 (Coker et al., 1997).

In normal lung tissues, TGF-β1 and -β3 are expressed in a variety of cells, most profoundly in bronchial epithelium, alveolar macrophages, mesenchymal, and endothelial cells (Coker et al., 1996). In bleomycin fibrosis, TGF-β1 was shown to be upregulated with maximal expression after 10 days. TGF-β1 mRNA is mainly produced by alveolar macrophages, and to a lesser extent mesenchymal and type II alveolar epithelial cells (Coker et al., 1997). In these studies, TGF-β3 mRNA was detectable in untreated mice at similar levels as TGF-β1, but in contrast to TGFβ1, bleomycin did not influence expression of TGF-β3. Earlier experiments investigating alveolar macrophages from bleomycin-treated rats have also shown that these cells secrete TGF-β1, but not TGF-β2 or -β3 (Khalil et al., 1993a; Khalil, Whitman, Zuo, Danielpour, & Greenberg, 1993). Another group failed to show differences in the expression of TGF-β isoforms after bleomycin (Santana et al., 1995). So far, only two studies have analyzed TGF-β3 expression in fibrotic human lung tissue, and both supported a more prominent role for TGF-β1 compared with TGF-β3 (Coker

et al., 2001; Khalil et al., 1996). More evidence supporting the hypothesis of TGF-β isoform imbalances in "fibrotic" versus "regular wound healing" can be found in other organ systems. The presence of TGF-β3 seems to promote scarless wound closure in the fetus, and recombinant TGF-β3 reduces scar formation in adult skin wounds, while TGF-β1 causes increased scarring (Shah et al., 1995). Pre-irradiation of wound beds results in diminished expression of TGF-β3, suggesting that a relative deficiency of TGF-β3 might be responsible for impaired wound healing in irradiated tissue (Schultze-Mosgau et al., 2003). Some reports, however, have been unable to demonstrate differences between the effects of TGF-β isoforms in cutaneous wounds (Wu et al., 1997). Investigations on intestinal myofibroblasts from patients with chronic inflammatory bowel disease have shown that TGF-β3 may be protective (McKaig et al., 2002, 2003). Intestinal myofibroblasts express mRNA of all three TGF-β isoforms, but secret mainly active TGF-β3, which enhances the migration of epithelial cells from the edges of wounds. Myofibroblasts derived from normal colon express mainly TGF-β3, those from patients with ulcerative colitis TGF-β1 and -β3 and are growth inhibited by antibody-mediated depletion of TGF-β. In contrast, myofibroblasts from patients with Crohn's disease express primarily TGF-β2 and almost no TGF-β3, and these cells are not growth-inhibited by antibodies to TGF-β (McKaig et al., 2002). In conclusion, these observations from other organ systems provide further support for the anti-scarring role of TGF-β3 in chronic inflammatory disease.

The hypothesis that the balance between TGF-β isoforms is an important component in wound healing was further addressed in this study by an experiment in which both TGF-β1 and -β3 were administered to rat lungs concomitantly. Addition of AdTGF-β3 blocked the AdTGF-β1-induced upregulation of TGF-β1, TIMP-1, fibronectin and CTGF by day 14, with sustained inhibition of TIMP-1 gene. This suggests a modulator role of TGF-β3 on TGF-β1 in this model. It is well established that TGF-β1 is capable of activating its own mRNA expression (Kim et al., 1990) which might explain the transient reduction of TGF-β1-responsive genes by 14 days, when TGF-β1 was still under "control" of TGF-β3. However, we were not able to demonstrate a substantial antifibrotic effect of TGF-β3 in the examined timeframe. This might be due to the complexity of the changes induced in the animal model and the fact that we are not able to accurately titrate the concentration and spatial distribution of transgenic TGF-β when using adenoviral gene transfer. We believe, however, that this data nevertheless strengthens the idea that a TGF-

β isoform balance skewed towards higher amounts of TGF-β3 might be of beneficial effect regarding wound healing.

It may be difficult to imagine why TGF-β1 and -β3 exhibit such distinct features *in vivo*, considering that both are supposed to similarly bind to the type II TGF-β receptor and transduce signals to various intracellular pathways (Eickelberg, 2001; Leask & Abraham, 2004). Ligand-binding studies are usually performed *in vitro*, and may vary widely depending on the cell line examined, and do not necessarily reflect the *in vivo* situation. Studies of TGF-β isoform knockouts strongly suggest distinct biological actions due to different phenotypes (reviewed in Ingman & Robertson, 2002). TGF-β1 null mutations show yolk sac defects with early embryonic death and chronic inflammation leading to perinatal mortality. In contrast, TGF-β3 null animals develop better, but die within few weeks after birth because of failure of palate fusion and bronchial branching abnormalities.

Recent research has demonstrated a wide range of TGF-β receptor subtypes (Derynck & Zhang, 2003). The binding of TGF-β is complicated by modulation through additional proteins—betaglycan, endoglin, and a pseudoreceptor named BAMBI. They all can bind TGF-β and either present it to signal-transducing receptors or prevent signalling by sequestering TGF-β. TGF-β signals through various intracellular pathways, the profibrotic mainly through Smad proteins. Recent evidence showed that non-Smad-signalling proteins, such as members of the activating protein-1 (AP-1 and JunD) and mitogen-activated protein kinase (MAPK) are also important for fibrogenesis (Eickelberg, 2001). In this study we analyzed expression of Smad proteins and found similarly increased phosphorylation of Smad2 and 3 upon overexpression of TGF-β1 and -β3 as expected. In contrast, the TGF-β type I receptor ALK 5 and type II receptor were more strongly induced following AdTGF-β1 expression. In line with the data presented in this paper, it has already been suggested that an increased TGFβ RI/RII ratio could promote fibrosis (Bock et al., 2005). The observation that TGF-β isoforms affect receptors differently, but not phosphorylation of Smad proteins suggests that non-Smad-signalling pathways may be involved in determining the final fibrotic response to TGF-β. Further analysis of Smad-independent pathways indicated reduced phosphorylation of AKT in AdTGF-β1-treated animals compared to AdTGF-β3. This pathway has been shown to be involved in the fibrotic response in other models (Martinez-Salgado, Fuentes-Calvo, Garcia-Cenador, Santos, & Lopez-Novoa, 2006), and a relative downregulation of AKT might promote fibrotic

tissue repair. Similarly, we observed some isoform-related differences in the levels of phospho-p44/42, which is part of another signalling cascade with cross-talk with the Smad pathway (Martinez-Salgado et al., 2006). We acknowledge that the differences in induction and phosphorylation of non-Smad-signalling proteins are subtle, but the observation nevertheless suggests that TGF-β isoforms 1 and 3 might affect tissue repair in a different manner due to influences on non-Smad-signalling pathways and TGF-β receptors. Our hypothesis for future studies is that the presence of a non-Smad-dependent mechanism in TGF-β1 signalling causes upregulation of TGF-β receptors, which is not manifest in TGF-β3 treated tissues. The progressive nature of TGF-β1-induced pulmonary fibrosis may be dependent on this regulatory loop rather than on differences in the Smad pathway.

In summary, we demonstrated that overexpression of TGF-β3 in rat lungs causes a transient and non-progressive fibrogenic response, which is strikingly different from TGF-β1. The progressive fibrogenesis in the lungs of AdTGF-β1-treated animals is associated with a prolonged upregulation of the TIMP-1 gene, which may result in reduced matrix degradation. We believe that these findings together with evidence from human tissue analysis and more extensive experimental work with skin wounds and chronic inflammatory bowel disease support our hypothesis that the balance between TGF-β isoforms is an important component in the progression of fibrotic wound healing. We further provide mechanistic explanations for this biological difference between TGF-β1 and -β3 by showing that TGF-β1 induces a stronger expression of the TGF-β type I receptor ALK 5 and the TGF-β type II receptor compared to TGF-β3, both on gene and protein levels. This suggests that the progressive fibroproliferation in is determined by the TGF-β type II and ALK 5 receptor, and less by changes in the Smad-signalling pathway.

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